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Prenatal Exposure to Mercury: Associations with Global DNA Methylation and Hydroxymethylation in Cord Blood and in Childhood

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Background: Mercury is a global pollutant, and prenatal exposure is associated with adverse health effects. To date, no studies have evaluated the association between prenatal mercury exposure and DNA hydroxymethylation, an epigenetic modification important for tissue differentiation and embryonic development.

Objectives: We sought to evaluate the association between prenatal mercury exposure and offspring global DNA methylation and hydroxymethylation at birth and test for persistence of the association in childhood.

Methods: Within Project Viva, a U.S. prebirth cohort, we examined associations of maternal second trimester red blood cell mercury (RBC-Hg) concentrations with global 5-hydroxymethylcytosine (%-5hmC) and 5-methylcytosine (%-5mC) DNA content in blood collected at birth ($n = 306$), early childhood ($n = 68$; 2.9 to 4.9 y), and midchildhood ($n = 260$; 6.7 to 10.5 y).

Results: Median prenatal RBC-Hg concentration was 3.23 μg/g [interquartile range (IQR) = 3.29]. At birth, median cord blood %-5mC, %-5hmC, and their ratio were 4.95%, 0.22%, and 24.37, respectively. The mean adjusted difference [95% confidence interval (CI)] of blood %-5hmC for a doubling in prenatal RBC-Hg concentration was −0.013% (−0.029, 0.002), −0.031% (−0.056, −0.006), and 0.005% (−0.007, 0.018) at birth, early, and midchildhood, respectively. The corresponding relative adjusted change in the genomic ratio of %-5mC to %-5hmC for a doubling in prenatal RBC-Hg concentration was 4.70% (0.04, 9.58), 22.42% (7.73, 39.11), and 0.73% (−4.18, 5.88) at birth, early, and midchildhood, respectively. No associations were present between prenatal maternal RBC-Hg and %-5mC at any time point.

Conclusions: Prenatal mercury exposure was associated with lower %-5hmC genomic content and a corresponding increase in the ratio of %-5mC to %-5hmC in cord blood. This association was persistent in early but not midchildhood blood. Our results demonstrate the potential malleability of epigenetic modifications associated with mercury exposure *in utero*. https://doi.org/10.1289/EHP1467

Introduction

Mercury is a global contaminant that bioaccumulates in the environment. Coal-burning emissions and industrial waste are among the major anthropogenic sources of mercury, and ocean mercury levels have tripled since the industrial revolution (Lamborg et al. 2014). Nonoccupational human exposures predominately occur through the consumption of fish and other seafood contaminated with methylmercury that has been biomagnified up the food chain (Driscoll et al. 2013). Methylmercury readily crosses the placenta and blood–brain barrier, exposing the fetus during critical windows of development (Stern and Smith 2003). Prenatal exposure to mercury, typical of regular fish consumption with elevated mercury content, has been associated with lower cognitive test scores and may also hinder infant growth in the first few years of life (Karagas et al. 2012). The specific mechanism of mercury toxicity remains poorly characterized, but several mechanisms, including oxidative stress, disruption of calcium homeostasis, and alterations of neurotransmitters, among others, are hypothesized to be involved (Castoldi et al. 2003). Recently, epidemiological studies have shown that prenatal mercury exposure is associated with DNA methylation at specific genomic regions (Bakulski et al. 2015; Cardenas et al. 2015) that could serve as a mechanism linking exposure and infant neurobehavioral outcomes (Maccani et al. 2015).

Epigenetic modifications during fetal development play a critical role during embryogenesis regulating cell lineage commitment, chromosome silencing, retrotransposon repression, and genetic imprinting (Cheng et al. 2015). One of the most widely studied epigenetic modifications is DNA methylation at the 5' position of cytosine (C) nucleotides, also referred to as 5-methylcytosine (5mC) (Relton et al. 2015). More recently, it has been demonstrated that 5mC can undergo oxidation by the ten-eleven translocation (TET) family of enzymes to form 5-hydroxymethylcytosine (5hmC) (Dao et al. 2014). Regulation of gene expression by 5hmC has been shown to be independent of 5mC and plays a crucial role during embryogenesis, particularly in neurogenesis (Etchegaray et al. 2015; Hahn et al. 2013). Similar to 5mC, genomic 5hmC is cell type–specific, and it is most abundant in neurons, embryonic stem cells, and pluripotent cells (Ruzov et al. 2011). While there is emerging literature on mercury exposure with DNA methylation and its potential role in neurodevelopment

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and fetal programming, we are not aware of any studies that have examined associations with 5hmC genomic content. Epigenetic measurements associated with prenatal mercury exposure might help elucidate mechanisms associated with toxicity, particularly for exposures occurring in the developing fetus during critical periods of epigenomic remodeling (Waterland and Michels 2007). Alternatively, unique epigenetic signatures could serve as biomarkers of exposure and be used as biosensors even years after the exposure window (Ladd-Acosta 2015). Identifying if epigenomic changes at birth and sustained throughout childhood are associated with prenatal exposure to mercury will help elucidate their potential role as biomarkers of exposure or disease susceptibility.

In the present analysis, we examined the association of prenatal maternal second trimester mercury exposure with global DNA 5hmC and 5mC content in cord blood. We tested for persistence of the association among blood DNA samples collected from children during early and midchildhood. We hypothesized that prenatal maternal mercury concentration would be associated with both global DNA methylation and hydroxymethylation, and the observed association would persist into early and midchildhood, reflecting persistent epigenetic reprogramming events occurring *in utero*.

Methods

Study Population

Mother–child pairs were participants in Project Viva, a prospective prebirth cohort study (Oken et al. 2014). This cohort was recruited between 1999 and 2002 during the mothers' first prenatal visits at Atrius Harvard Vanguard Medical Associates, a multispecialty medical group practice in Massachusetts, United States. Eligibility criteria included fluency in English, gestational age less than 22 wk at the first prenatal visit, and singleton pregnancy. Of the total 2,128 live births, cord blood was collected from 1,018 born at one of the two study hospitals. We measured global genomic 5hmC and 5mC content in 481 cord blood samples for mothers who provided consent for genetic analysis. Of these, 306 samples had complete covariate information and maternal prenatal blood mercury concentrations. We evaluated persistence of cord blood associations in 68 children with available blood epigenetic measurements in early childhood (age: 2.9 to 4.9 y) and 260 children with blood epigenetic measurements from midchildhood (age: 6.7 to 10.5 y) with complete exposure and covariate information. Not all participants were measured at all three time points: of the 306 infants eligible for analyses in cord blood, 63 provided early childhood blood, and 144 provided samples for midchildhood analyses after restricting on complete covariate information. We did not restrict our analyses to participants with repeated epigenomic measurements at all three time points in order to maximize the sample size at each time point, but instead performed a sensitivity analyses among repeated measurements (sample flow diagram is depicted in Figure S1). Mothers provided written informed consent at recruitment and at postpartum follow-up visits. The Institutional Review Board of Harvard Pilgrim Health Care reviewed and approved all study protocols.

Maternal Prenatal Red Blood Cell Mercury

Sample collection and exposure assessment for prenatal mercury exposure in this cohort has been previously described (Oken et al. 2016). Briefly, at the second trimester visit, we obtained a maternal blood sample that was centrifuged to separate plasma from blood erythrocytes. A separate aliquot of erythrocytes was provided for mercury analysis. Aliquots were stored at −70°C and analyzed for total mercury using the Direct Mercury Analyzer 80 (Milestone Inc.). Results are reported as mercury concentration in the original red blood cell sample in ng/g. The detection limit was 0.5 ng/g of sample, and the percentage recovery for standards ranged from 90% to 110%. A total of 32 samples were below the limit of detection (LOD), and we used the instrument estimated red blood cell mercury (RBC-Hg) concentration below the LOD for analyses. During pregnancy, participants also completed a semiquantitative food frequency questionnaire (FFQ) that we previously calibrated against erythrocyte levels of elongated $n-3$ fatty acids (Fawzi et al. 2004). Participants self-reported their consumption of fish with six frequency response options ranging from "never/less than 1 per month" to "1 or more servings per day." We combined responses to estimate average total fish intake as average servings per week for the first and the second trimesters.

DNA Methylation and Hydroxymethylation

We isolated 1 μg of DNA from buffy coat and enzymatically hydrolyzed it to individual deoxyribonucleosides by a simple, one-step DNA hydrolysis procedure consisting of a digest mix prepared by adding phosphodiesterase I, alkaline phosphatase, and benzonase Nuclease to Tris-HCl buffer (Sigma-Aldrich, Diegem, Belgium). We spiked extracted DNA with an internal standard mixture, dried it, and then hydrolyzed at 37°C for at least 8 h with 10 μL digest mixture. After hydrolysis, we added 490 μL ACN (acetonitrile): H_2O (90:8, v/v) to each sample. We prepared stock solutions of 5mC, 5hmC, and cytosine (C) by dissolving commercial standards (Sigma-Aldrich) in 22 high-performance liquid chromatography–grade H_2O to prepare the calibration standards. Finally, we measured global genomic DNA methylation and hydroxymethylation of cytosine nucleotides simultaneously for each sample using ultrapressure liquid-chromatography combined with tandem mass spectrometry, as previously described (Godderis et al. 2015). The lower limits of detection were 0.096 ng/mL, 0.008 ng/mL, and 0.62 ng/mL for 5mC, 5hmC, and C, respectively. The accuracy for the concentrations tested ranged from 87% to 114.8% of the target, and the precision expressed as relative standard errors ranged from 0.8% to 12.1%. Global DNA methylation is reported as a percentage of 5mC over the sum of 5mC, 5hmC, and C [$\%-5mC = 5mC/(5mC + 5hmC + C)$], while global DNA hydroxymethylation is expressed as a percentage of 5hmC versus the sum of 5mC, 5hmC, and C [$\%–5hmC = 5hmC/(5mC + 5hmC + C)$]. A ratio of %-5mC to %-5hmC is also reported (%-5mC/%–5hmC). All samples analyzed were above the LOD. However, a total of 24 samples, 11 in cord blood, 4 in early, and 9 in midchildhood were below the limit of quantification and therefore excluded from analyses. For further details on the methodology, see the supplementary materials on the methods that include analytical conditions, calibration curves, and validation parameters for our method (Tables S3–S4 and Figure S7).

Covariates

At study enrollment, we obtained information on maternal race/ethnicity, education, prepregnancy body mass index, smoking during pregnancy, marital status, parity, age, and household income. Infant sex, birth weight, and type of delivery were abstracted from medical records. We determined birth weight for gestational age and sex z-score from a U.S. national reference (Oken et al. 2003). Infant race/ethnicity was collected by interview. We obtained data on micronutrient intake using self-administered semiquantitative FFQ during the first and second trimesters that were previously validated and modified to be used

in pregnancy (Fawzi et al. 2004). We evaluated the effect of adjusting for intake of four methyl donors (vitamin B-12, folate, betaine, and choline) estimated from FFQs in the first or second trimester of pregnancy, given that one carbon metabolism plays a central role in DNA methylation and prenatal maternal methyl donor intake might play a role in fetal programming of the epigenome (Pauwels et al. 2016).

Statistical Analyses

We estimated medians and interquartile ranges (IQRs) for %-5hmC, %-5mC, and their ratio in cord blood across demographic and prenatal nutritional information for the study population among all mother–child pairs available with epigenomic measurements at birth without restricting on prenatal mercury exposure ($n = 481$). We tested unadjusted associations for %-5hmC, %-5mC, and their ratio with participant characteristics with two levels using a nonparametric Wilcoxon-rank sum test, and use the Kruskal-Wallis test for characteristics with more than two levels. We used Spearman correlation coefficients to estimate the association of %-5hmC and %-5mC in cord blood with levels in blood during early or midchildhood. We also evaluated changes in %-5mC, %-5hmC, and their ratio among subjects with repeated measurements from cord blood and in early ($n = 63$) or cord blood and midchildhood ($n = 144$) using Wilcoxon signed-rank test for paired samples.

Both RBC-Hg concentrations and the ratio of %-5mC and %-5hmC were right skewed. Therefore, we $\log_2$-transformed them to approximate a normal distribution. We used separate linear regression models to estimate associations between $\log_2$-transformed prenatal maternal RBC-Hg and %-5hmC, %-5mC, and the $\log_2$-transformed ratio of %-5mC to %-5hmC in cord blood as well as in blood collected during early and midchildhood. The ratio of %-5mC to %-5hmC was modeled separately as a potential target for DNA demethylation.

We estimated unadjusted as well as adjusted associations using two multivariate linear regression models: Model 1, a linear regression model adjusted for covariates selected *a priori* that were nominally associated with the outcome in univariate analyses ($p < 0.1$) or that change the effect estimate for mercury by at least 10% regardless of statistical significance. Model 1 was adjusted for maternal education, age at enrollment, marital status, first trimester vitamin B-12 intake, and second trimester fish consumption, as well as child race/ethnicity, sex, gestational age, and birth weight for gestational age *z*-score. The %-5mC model was further adjusted for first trimester folate intake, while the %-5hmC model was adjusted by first trimester betaine intake. The model for the ratio of %-5mC to %-5hmC was adjusted for both first trimester folate and betaine intake. Model 2 was adjusted for the same covariates as Model 1, but also included estimated cell type proportions. Cell type composition was estimated from genome-wide DNA methylation arrays (HumanMethylation450 BeadChip; Illumina) available in a subset of samples and estimated using the minfi package of R (version 3.3.0, R Core Team) (Aryee et al. 2014). We used an adult reference DNA methylation panel to estimate leukocyte composition in early and midchildhood and a cord blood DNA methylation reference panel, which includes nucleated red blood cells (nRBCs), to estimate nucleated cell types at birth (Bakulski et al. 2016). Associations between cell type composition in cord blood and both epigenomic measures are summarized in Table S1. We report the relative percent change in the ratio of 5mC to 5hmC per doubling in exposure, as regression coefficients were estimated from linear models where RBC-Hg and the ratio of %-5mC and %-5hmC were both $\log_2$-transformed (log–log adjusted models) to meet model assumptions. Unadjusted and fully adjusted models were restricted to complete covariate information used in fully adjusted models in order to compare models.

We evaluated persistence by testing associations between prenatal maternal RBC-Hg concentration and epigenetic measurements among all participants who provided blood samples during early or midchildhood. Estimates from unadjusted and adjusted models are reported. Early and midchildhood adjusted linear regression models were similar to Model 2 for cord blood with the addition of child age in days at blood collection and replacing cell type composition with the estimated leukocyte distribution obtained from genome-wide DNA methylation arrays measured during early or midchildhood. We evaluated effect modification by testing the interaction between sex and RBC-Hg for all epigenomic measures across time points. Model fit and assumptions were examined using scatterplots of the standardized residuals vs. the fitted values, residuals vs. the leverage, and quantile-quantile plots of the standardized residuals. All analyses were performed using R 3.3.0 (www.r-project.org).

Results

Sociodemographic and Unadjusted Associations

A total of 481 participants with measurements of cord blood DNA %-5mC and %-5hmC were available for bivariate analyses. The median %-5hmC content for the entire sample was 0.22% (IQR = 0.19) (min–max: 0.024-0.87; skewness: 1.04) and the median %-5mC was 4.95% (IQR = 2.52) (min–max: 2.24–10.81; skewness: 0.94). The median ratio of %-5mC to %-5hmC in cord blood was 24.37 (IQR = 14.89). In unadjusted analyses, median %-5hmC and %-5mC in cord blood varied by participant characteristics (Table 1).

In unadjusted analyses, prenatal RBC-Hg concentration measured during the second trimester of pregnancy was associated with global %-5hmC content measured in cord blood (Table 1). Namely, the lowest cord blood 5hmC content was observed among participants in the highest prenatal exposure quartile (0.18%) compared to those in the third (0.20%), second (0.25%), or first (0.20%) quartiles of exposure. The ratio of 5mC to 5hmC was higher in the highest prenatal mercury exposure quartile (27.9) compared to the third (23.9), second (23.1), or first (23.7) quartiles of exposure.

Correlations among Epigenomic Measurements and Longitudinal Changes

Among all participants with epigenomic measurements, global %-5hmC and %-5mC were strongly correlated in cord blood ($r_s = 0.75$; $p < 2.2 \times 10^{-16}$), as well as in blood collected during early ($r_s = 0.70$; $p < 2.2 \times 10^{-16}$) and in midchildhood ($r_s = 0.72$; $p < 2.2 \times 10^{-16}$), illustrated in Figure 1.

Among participants with repeated epigenomic measurements ($n = 111$), median cord blood %-5hmC was higher (0.22%) compared to median %-5hmC measured in blood during early childhood (0.15%; $p = 1.7 \times 10^{-6}$). Similarly, median %-5mC was higher in cord blood (5.06%) compared to the median %-5mC measured during early childhood (4.63%; $p = 2.5 \times 10^{-5}$). Correspondingly, among participants with repeated epigenomic measurements in cord blood and midchildhood ($n = 225$), median cord blood %-5hmC was higher (0.24%) compared to median levels of %-5hmC measured in blood during midchildhood (0.16%; $p = 3.9 \times 10^{-10}$), as well as levels of %-5mC in cord blood (5.52%) compared to measurements in midchildhood blood (5.31%; $p = 3.0 \times 10^{-5}$).

However, median levels of %-5hmC measured during early (0.14%) and midchildhood (0.13%) ($n = 61$; $p = 0.86$) and the

Table 1. Sample characteristics of 481 mother–child pairs in the Project Viva cohort and distribution of cord blood DNA 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC) by these characteristics.

		%-5hmC [median (IQR)]		%-5mC [median (IQR)]		5mC to 5hmC ratio [median (IQR)]	
Characteristics	%	$N = 481$	p-Value	$N = 481$	p-Value	$N = 481$	p-Value
Overall		0.22% (0.19)	—	4.95% (2.52)	—	24.37 (14.89)	—
Maternal race/ethnicity							
White	71.9	0.22 (0.19)	0.62	4.95 (2.49)		24.8 (15.4)	0.91
Black	11.9	0.23 (0.15)		4.94 (2.06)	0.09	23.5 (15.5)	
Hispanic	6.9	0.24 (0.19)		6.31 (3.02)		23.5 (13.2)	
Other	9.4	0.21 (0.15)		4.58 (1.99)		22.7 (10.2)	
Prepregnancy BMI (kg/m^2)							
Underweight (<18.5)	3.5	0.19 (0.19)	0.91	5.37 (3.16)	0.63	28.5 (18.3)	0.89
Normal (18.5 – < 25.0)	60.2	0.21 (0.19)		4.88 (2.64)		24.2 (14.8)	
Overweight (25.0 – < 30.0)	22.1	0.23 (0.20)		5.15 (2.56)		24.6 (14.4)	
Obese (≥30.0)	14.2	0.23 (0.14)		4.88 (2.11)		23.1 (14.2)	
Missing	$n = 1$	—		—		—	
College graduate							
No	33.5	0.24 (0.22)	0.03	5.14 (2.59)	0.03	23.3 (14.1)	0.13
Yes	66.5	0.20 (0.18)		4.82 (2.41)		24.7 (15.1)	
Self-reported Smoking							
Never	68.6	0.23 (0.18)	0.68	5.00 (2.49)	0.30	23.7 (15.0)	0.89
Former	20.8	0.20 (0.21)		4.70 (2.78)		25.4 (14.2)	
During pregnancy	10.6	0.20 (0.17)		4.95 (1.80)		23.9 (14.7)	
Nulliparous							
No	53.8	0.23 (0.19)	0.71	4.82 (2.40)	0.87	24.1 (14.8)	0.72
Yes	46.2	0.21 (0.18)		5.07 (2.55)		24.5 (14.6)	
Any alcohol intake (first trimester)							
No	25.2	0.24 (0.19)	0.38	5.11 (2.32)	0.84	24.4 (13.0)	0.45
Yes	74.8	0.22 (0.19)		4.95 (2.54)		24.5 (15.5)	
Missing	$n = 40$	—		—		—	
Maternal age at enrollment (y)							
15.7–29.6	25.2	0.23 (0.20)	0.75	5.31 (2.49)	0.56	23.8 (13.9)	0.65
>29.6–32.4	24.5	0.20 (0.19)		4.87 (2.46)		24.6 (14.5)	
>32.4–35.8	25.2	0.23 (0.18)		4.71 (2.37)		23.7 (12.2)	
>35.8–44.9	25.2	0.22 (0.19)		4.94 (2.68)		24.4 (17.8)	
Married or cohabitating							
No	9.1	0.27 (0.26)	0.03	5.84 (3.61)	0.08	22.6 (15.3)	0.24
Yes	90.9	0.22 (0.18)		4.88 (2.43)		24.5 (15.0)	
Household income >$70,000							
No	40	0.23 (0.22)	0.83	4.90 (2.54)	0.65	24.8 (17.1)	0.95
Yes	60	0.22 (0.17)		5.04 (2.54)		24.2 (13.5)	
Missing	$n = 36$	—		—		—	
First trimester fish (serving/wk)							
0	10.9	0.25 (0.16)	0.10	5.08 (2.13)	0.71	22.4 (12.6)	0.03
>0– < 3.0	75.7	0.23 (0.20)		4.97 (2.50)		24.0 (15.4)	
≥3.0	13.4	0.19 (0.12)		5.98 (2.40)		28.0 (12.5)	
Missing	$n = 40$	—		—		—	
Second trimester fish (serving/wk)							
0	13.2	0.24 (0.21)	0.06	5.03 (2.42)	0.94	22.6 (13.6)	0.01
>0– < 3.0	75.6	0.23 (0.20)		4.95 (2.46)		24.2 (14.2)	
≥3.0	11.2	0.17 (0.14)		4.88 (3.66)		30.7 (19.2)	
Missing	$n = 63$	—		—		—	
2nd trimester RBC-Hg (ng/g)							
0.15–1.65	25.2	0.20 (0.18)	0.030	4.71 (2.37)	0.88	23.7 (15.3)	0.008
>1.65–3.23	24.2	0.25 (0.23)		5.27 (2.77)		23.1 (14.3)	
>3.23–4.94	24.2	0.20 (0.22)		4.72 (2.84)		23.9 (14.4)	
>4.94–24.5	25.2	0.18 (0.15)		5.00 (2.43)		27.8 (16.2)	
Missing	$n = 159$	—		—		—	
Child sex							
Male	53.4	0.22 (0.18)	0.61	4.83 (2.46)	0.42	24.5 (15.3)	0.74
Female	46.6	0.22 (0.19)		5.05 (2.49)		23.7 (14.3)	
Child race/ethnicity							
White	67.8	0.22 (0.19)	0.84	4.94 (2.46)	0.03	24.6 (15.5)	0.59
Black	12.9	0.23 (0.16)		4.83 (2.06)		23.1 (14.7)	
Hispanic	4.8	0.24 (0.19)		6.84 (3.91)		26.0 (18.6)	
Other	14.6	0.22 (0.17)		4.71 (2.50)		24.2 (11.2)	
C-section birth							
No	83.4	0.22 (0.18)	0.41	4.95 (2.41)	0.49	24.1 (13.7)	0.31
Yes	16.6	0.20 (0.20)		4.72 (2.84)		26.8 (15.3)	

Table 1. (*Continued.*)

Characteristics	%	%-5hmC [median (IQR)]		%-5mC [median (IQR)]		5mC to 5hmC ratio [median (IQR)]	
		$N = 481$	p-Value	$N = 481$	p-Value	$N = 481$	p-Value
Gestation length <37 wks							
No	95.6	0.22 (0.19)	0.69	4.93 (2.43)	0.37	24.3 (15.2)	0.93
Yes	4.4	0.23 (0.21)		6.38 (3.39)		25.1 (7.5)	
BW/GA category							
Small for gestational age (SGA) <10th percentile	4.4	0.18 (0.06)	0.10	4.62 (2.07)	0.53	29.5 (11.8)	0.05
Appropriate for gestational age 10–90	80.9	0.23 (0.19)		5.00 (2.56)		23.7 (13.9)	
Large for gestational age (LGA) >90	14.8	0.20 (0.16)		4.85 (2.57)		26.0 (16.0)	

Note: BMI, body mass index; BW, body weight; IQR, interquartile range. p-Value calculated from a Kruskal-Wallis test for groups with more than two levels and a Wilcoxon-rank sum test for groups with only two levels.

corresponding levels of %-5mC genomic content (4.57% and 4.42%, $p = 0.96$) were similar. A similar trend of elevated %-5hmC and %-5mC in cord blood compared to early or midchildhood blood was observed among all participants (Figure S2).

Association of 5-Hydroxymethylcytosine and 5-Methylcytosine with Prenatal Red Blood Cell Mercury Concentrations

In unadjusted linear regression analyses, a doubling in prenatal mercury exposure was associated with lower global %-5hmC [$\beta = -0.013$; 95% confidence interval (CI): −0.026, 0.000; $p = 0.05$] and an increase in the ratio of 5mC to 5hmC ($\beta = 5.70\%$; 95% CI: 1.60, 9.95; $p = 0.006$) in cord blood. A doubling in RBC-Hg concentration was associated with a 0.015% mean decrease in global 5hmC content (95% CI: −0.029, 0.0003; $p = 0.06$) after adjusting for maternal education, age at enrollment, marital status, first trimester vitamin B-12, first trimester betaine intake, second trimester fish consumption, and child race/ethnicity, sex, gestational age, and birth weight for gestational age z-scores (Table 2). After further adjustment for the estimated cell type composition, this association was slightly attenuated ($\beta = -0.013$; 95% CI: −0.029, 0.002; $p = 0.09$), Table 2. Correspondingly, a doubling in prenatal mercury exposure was associated with a 4.80% increase in the ratio of %-5mC to %-5hmC (95% CI: 0.18, 9.69; $p = 0.041$) after adjusting for potential confounders. This association remained consistent after further adjusting for the estimated cell type composition of cord blood ($\beta = 4.70\%$; 95% CI: 0.04, 9.58; $p = 0.047$). In this sample, no associations were observed with %-5mC in cord blood and prenatal mercury exposure (Table 2).

In early childhood (age: 2.9 to 4.9 y), a doubling in RBC-Hg concentration was associated with a 0.031% mean decrease in global %-5hmC measured in blood ($\beta = -0.031$; 95% CI: −0.056, −0.006; $p = 0.016$) after adjusting for covariates (Table 3). Correspondingly, in fully adjusted models, a doubling in prenatal RBC-Hg concentration was associated with a 22.42% increase in the ratio of %-5mC to %-5hmC in early childhood (95% CI: 7.73, 39.11; $p = 2.58 \times 10^{-3}$). Prenatal RBC-Hg concentration was not associated with %-5mC in early childhood. No associations were observed between prenatal RBC-Hg concentration and

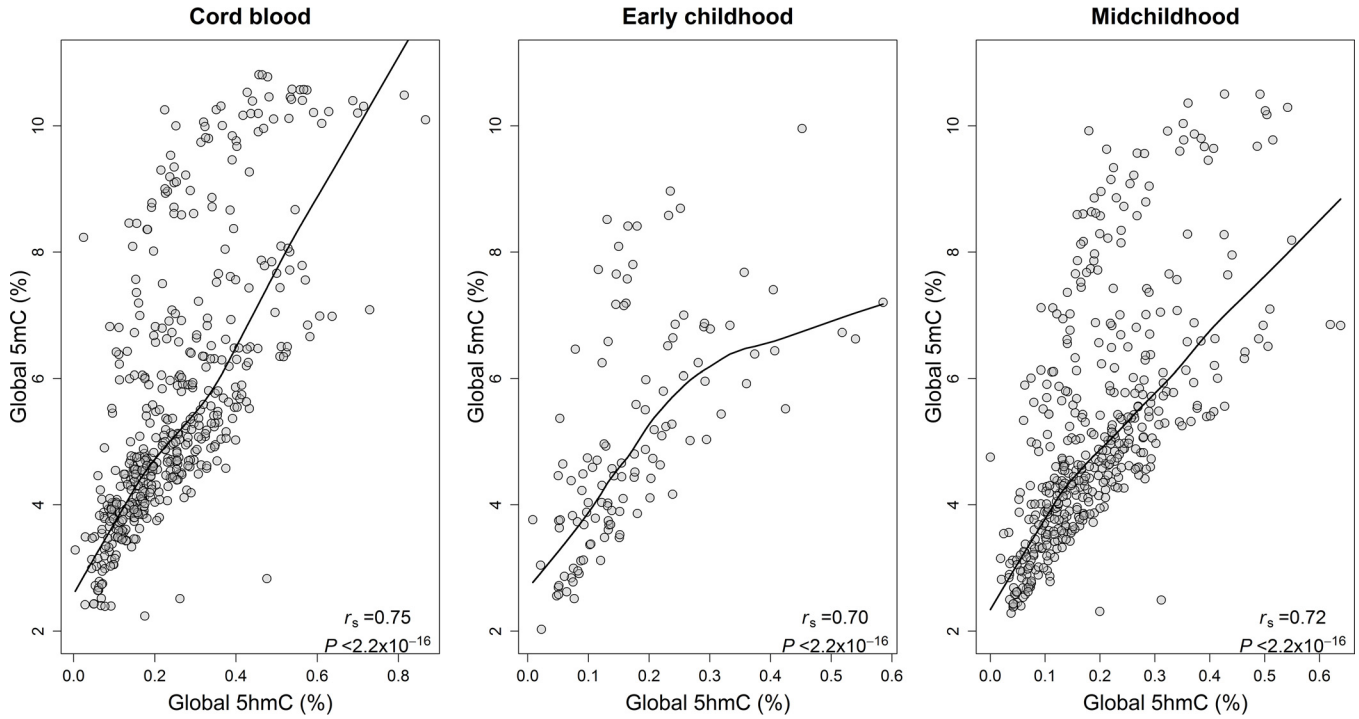

Figure 1. *Scatter plots, Spearman correlations coefficients and locally weighted scatterplot smoothing lines for 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC) at three sample collection periods: at birth (cord blood, $n = 481$), early childhood ($n = 117$), and midchildhood blood ($n = 444$).
Note: All participants with blood epigenomic measurements and not restricted to prenatal mercury exposure.

Table 2. Percent change in global 5-hydroxymethylcytosine (5hmC), global 5-methylcytosine (5mC), and their ratio in cord blood per doubling in prenatal maternal red blood cell mercury (RBC-Hg) concentrations.

Model	n	Change in global %-5hmC (95% CI)	p-Value	Change in global %-5mC (95% CI)	p-Value	%-change in the ratio of 5mC to 5hmC (95% CI)	p-Value
Unadjusted	306	−0.013% (−0.026, 0.000)	0.05	−0.029% (−0.209, 0.149)	0.74	5.70% (1.60, 9.95)	0.006
*Model 1	306	−0.015% (−0.029, 0.000)	0.06	−0.074% (−0.278, 0.128)	0.47	4.80% (0.18, 9.69)	0.04
†Model 2	306	−0.013% (−0.029, 0.002)	0.09	−0.049% (−0.251, 0.151)	0.63	4.70% (0.04, 9.58)	0.05

*Model 1 adjusted for maternal education, age at enrollment, marital status, first trimester vitamin B-12 intake, second trimester fish consumption, and child race/ethnicity, sex, gestational age, and birth weight for gestational age z-scores. Additionally, the 5-hydroxymethylcytosine (%-5hmC) model was further adjusted for first trimester betaine intake, while the 5-methylcytosine (%-5mC) model was further adjusted for first trimester folate intake. The model for the ratio of 5mC to 5hmC was adjusted for both betaine and folate intake during the first trimester.
†Model 2: Model 1 + six estimated cell types [($CD8^+$ T cells, $CD4^+$ T cells, Natural killer cells, monocytes, granulocytes, and nucleated red blood cells (nRBCs)].

global %-5hmC, %-5mC, or their ratio measured during midchildhood in unadjusted or fully adjusted models (Table 3). Fully adjusted estimates of the associations of prenatal RBC-Hg concentration with epigenomic measures at all measured time points are shown in Figure 2. In sensitivity analyses restricted to participants with repeated epigenomic measurements available in cord blood and in early childhood ($n = 65$) or cord blood and midchildhood ($n = 144$) yielded consistent results (Figure S3). Analyses stratified by gender suggested that the association might be stronger for females. However, in fully adjusted models, there was no evidence of a significant interaction by sex for the association between prenatal mercury exposure and global %-5hmC at birth ($p_{interaction} = 0.42$) or in early childhood ($p_{interaction} = 0.73$). Similarly, the association between prenatal mercury exposure and the ratio of 5mC to 5hmC at birth was not significantly different by gender ($p_{interaction} = 0.89$) or in early childhood ($p_{interaction} = 0.98$). Adjustment for maternal smoking, either sustained during pregnancy or any smoking during pregnancy, did not influence the results. Furthermore, we observed similar results when samples below the LOD were replaced by the LOD/$\sqrt{2}$ (results not shown).

Discussion

In this U.S. prebirth cohort, higher prenatal maternal mercury exposure measured during the second trimester of pregnancy was associated with lower global %-5hmC DNA content in cord blood after adjusting for potential confounders. This association was persistent in early (2.9 to 4.9 y), but not in midchildhood (6.7 to 10.5 y). Correspondingly, prenatal mercury exposure was associated with an increase in the ratio of %-5mC to %-5hmC in cord blood, also persisting into early but not midchildhood. No associations between prenatal maternal red blood cell mercury concentrations and global %-5mC DNA content were evident. Global 5hmC and 5mC were higher in cord blood compared to childhood blood samples. Additionally, both 5hmC and 5mC were positively correlated with each other in cord blood in early and midchildhood blood. Our study highlights the potential toxicity of prenatal mercury exposure during fetal epigenetic reprogramming and suggests that the association might be reversible during childhood.

Although 5hmC is an epigenetic modification known to be important during embryogenesis, little is known about its physiological role and predictors. The distribution of 5hmC has been shown to be a relatively stable epigenetic mark *in vitro* and in animal models, and not just present as a transient demethylation product, likely playing an independent regulatory function regulation (Bachman et al. 2014; Iurlaro et al. 2013; Spruijt et al. 2013). For example, stem cell models have shown that enhancer regions in the genome have greater abundance of 5hmC having a repressive distal role on transcription (Choi et al. 2014; Yu et al. 2012). In embryonic stem cells, 5hmC plays a dual role by maintaining chromatin structure for both upregulated genes upon differentiation and for polycomb group repressed genes (Pastor et al. 2011; Wu et al. 2011). Disruption of 5hmC levels by knocking down TET2 function has been shown to affect hematopoietic cell differentiation, a potential contributor to myeloid malignancies, highlighting the important role of 5hmC levels in blood (Pronier et al. 2011).

Few studies have examined fetal 5hmC disruption during human development. For example, among cases of preeclampsia and gestational diabetes, both conditions known to affect the intrauterine environment, 5hmC content and TET2 expression in umbilical vein tissue were observed to be altered compared to controls, suggesting that an adverse intrauterine environment can influence 5hmC during development (Sun et al. 2016). In this same study, it was shown with cell cultures that hypoxia is a potential mechanism for the observed alteration in 5hmC levels. Furthermore, oxidative stress has been shown to alter the distribution of 5hmC at several genomic regions in both cell cultures and *in vivo*. For example, inducing oxidative stress led to a global decrease in hydroxymethylation *in vitro* and *in vivo* in the epithelium of a double-knockout animal model deficient in glutathione peroxidases 1 and 2, major antioxidant enzymes (Delatte et al. 2015). Consistent with these models, we also

Table 3. Percent change in global 5-hydroxymethylcytosine (5hmC), global 5-methylcytosine (5mC), and their ratio in blood collected early and midchildhood per doubling in prenatal maternal red blood cell mercury (RBC-Hg) concentrations.

Childhood time point	n	Change in global %-5hmC (95% CI)	p-Value	Change in global %-5mC (95% CI)	p-Value	% change in the ratio of 5mC to 5hmC (95% CI)	p-Value
Early childhood (2.9 to 4.9 y)							
Unadjusted	68	−0.015% (−0.035, 0.005)	0.15	−0.022% (−0.345, 0.300)	0.89	10.69% (0.10, 22.39)	0.05
Adjusted[a]	68	−0.031% (−0.056, −0.006)	0.02	−0.057% (−0.451, 0.337)	0.77	22.42% (7.73, 39.11)	2.58×10^{-3}
Midchildhood (6.7 to 10.5 y)							
Unadjusted	260	0.002% (−0.009, 0.013)	0.77	0.010% (−0.172, 0.192)	0.91	1.00% (−3.32, 5.51)	0.65
Adjusted[a]	260	0.005% (−0.007, 0.018)	0.41	0.094% (−0.113, 0.301)	0.37	0.73% (−4.18, 5.88)	0.78

[a]Linear regression models adjusted for maternal education, age at enrollment, marital status, first trimester vitamin B-12 intake, second trimester fish consumption, and child race/ethnicity, sex, gestational age, birth weight for gestational age z-scores, child age at blood collection, and estimated leukocyte composition in early or midchildhood ($CD8^+$ T cells, $CD4^+$ T cells, Natural killer cells, monocytes, and granulocytes). Additionally, the %-5hmC model was further adjusted for first trimester betaine intake, while the %-5mC model was further adjusted for first trimester folate intake. The model for the ratio of 5mC to 5hmC was adjusted for both betaine and folate intake during the first trimester.

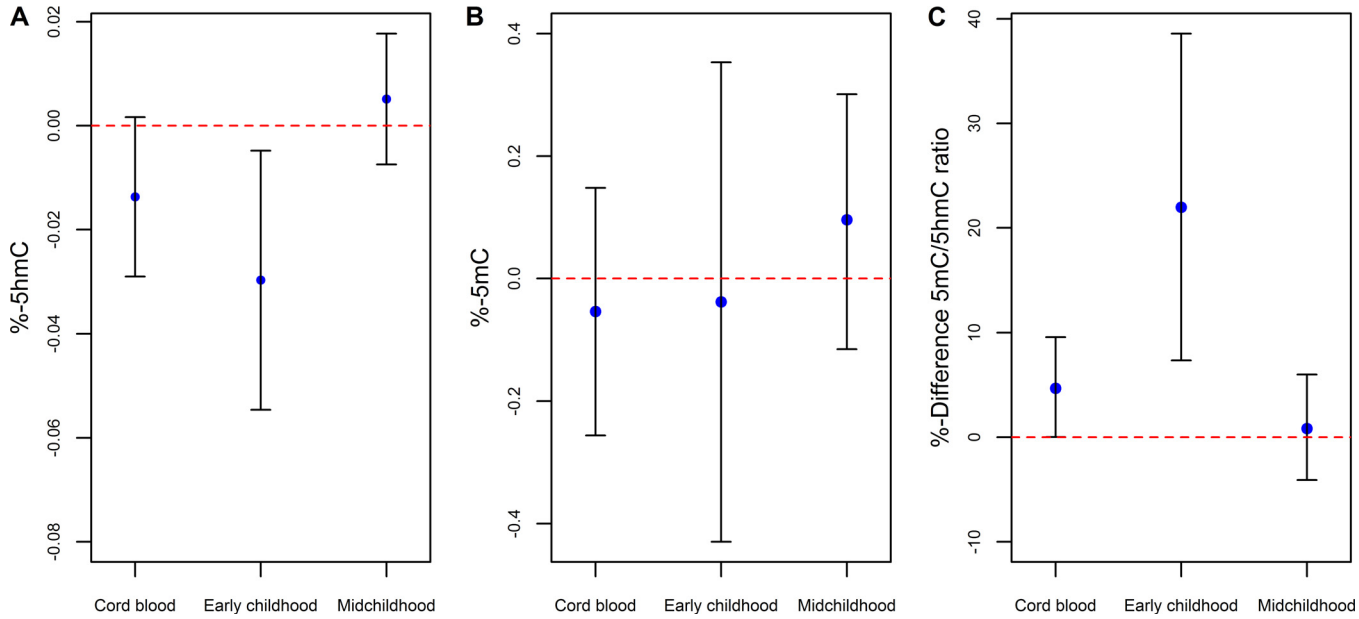

Figure 2. Fully adjusted association for *A*) 5-hydroxymethylcytosine (%-5hmC); *B*) 5-methylcytosine (%-5mC;) and *C*) %-difference in the ratio of 5mC to 5hmC per doubling in prenatal maternal mercury concentrations.

observed a decrease in global measurements of 5hmC in the cord blood associated with increasing levels of prenatal mercury exposure. Interestingly, the leading hypothesized mechanisms for mercury-associated toxicity include the generation of reactive oxygen species and increased oxidative stress (Farina et al. 2011a; Farina et al. 2011b). Moreover, metal-induced oxidative stress is also thought to be a unifying process for epigenetic disruption (Baccarelli and Bollati 2009; Hou et al. 2012). Oxidative stress or antioxidant depletion might be involved in the observed association between prenatal mercury exposure and lower 5hmC content. Future experimental studies could test this hypothesis.

A few studies have shown that 5hmC in blood might be sensitive to environmental exposures. In a cross-sectional study, in adult participants from the Strong Heart Study (American Indians from Arizona, Oklahoma, North Dakota, and South Dakota; 45 to 74 y old), higher cadmium and arsenic exposure were each associated with higher %-5hmC of blood DNA (Tellez-Plaza et al. 2014). This study also reported moderate correlations (r_s: 0.32 to 0.54) for the relationship between 5hmC and 5mC. Likewise, another study of adults reported similar Pearson correlations between 5hmC and 5mC measured in blood at different time points ($r = 0.35$ to 0.22) and an increase in %-5hmC with elevated particulate matter $\leq 10\,\mu m$ (PM_{10}) exposure (Sanchez-Guerra et al. 2015). Although these two studies reported lower correlations among epigenomic measures (5hmC and 5mC) compared to our data, their target populations were adults, and both studies used an enzyme-linked immunosorbent assay commercial method for the quantification of 5mC and 5hmC that differs from our mass spectrometry methodology. In these two previous studies, arsenic and PM_{10} exposure were positively associated with %-5hmC content, but not %-5mC. We observed an inverse association between prenatal mercury exposure and %-5hmC, but the association with %-5mC was null, suggesting that 5hmC might be a more sensitive biomarker of environmental exposures. Moreover, the observed increase in the ratio of 5mC to 5hmC paired with the null associations for 5mC suggests that prenatal mercury exposure does not influence the demethylation pathway of 5mC to the 5hmC product. Instead, our results suggest that the 5hmC association with prenatal maternal mercury exposure is independent of 5mC levels in the genome. The health implications of the associations between metals and %-5hmC in both children and adults are currently unknown. Prospective epidemiological and molecular studies with metal exposure assessment, epigenetic measures that distinguish 5hmC from 5mC, and health end points over the follow-up are needed.

Several studies have examined the association between prenatal mercury exposure and DNA methylation across the genome. Namely, a previous epigenome-wide association study of mother–infant pairs documented hypermethylation of top CpG sites along with shifts in cell type composition of cord blood relative to maternal methylmercury concentrations in a population exposed to low levels of mercury mainly through the diet (Cardenas et al. 2015). Another study of mother–infant pairs likely exposed through dietary sources documented methylation changes of a genomic region associated with both total prenatal mercury and methylmercury exposure, but the association was attenuated after adjusting for cell type composition (Bakulski et al. 2015). In a study of dental health professionals occupationally exposed to mercury, a cross-sectional relationship was observed between methylmercury concentration and hypomethylation of a candidate gene (*SEPP1*), but no relationship with global DNA methylation of repetitive elements such as Long Interspersed Element (LINE-1) (Goodrich et al. 2013). In an epigenome-wide association study of our cohort, we observed that prenatal maternal RBC-Hg concentration was associated with regional DNA methylation of the *PON1* gene in cord blood for males, an association that persisted in early childhood but was attenuated in midchildhood, supporting our findings of the potential malleability of the epigenetic modifications early in life (Cardenas et al. 2017). None of the gene-specific CpGs previously reported to be associated with prenatal mercury exposure in our cohort were significantly correlated with global measures of DNA methylation or DNA hydroxymethylation (Figure S4). Although *PON1* cord blood DNA methylation was associated with child cognitive function in early childhood in our previous Epigenome Wide Association Study (EWAS) (Cardenas et al. 2017), we did not observe an association for global %-5mC and

%-5hmC measured in cord blood or early childhood with child cognitive function (Table S2). Additionally, we tested whether DNA methylation measurements from the Illumina Infinium HumanMethylation450 BeadChip or 450K array were associated with our global measurements of %-5hmC and %-5mC among the same samples using the Global Analysis of Methylation Profiles package of R (Zhao et al. 2015). Our global measures of DNA %-5hmC were not associated with the cumulative density function ($p = 0.18$) or the density distribution of the 450K measurements ($p = 0.10$). Furthermore, our global measurements of DNA %-5mC was not significantly associated with either the cumulative density function ($p = 0.11$) or the density distribution of the 450K measurements ($p = 0.14$). As sensitivity analyses, we also examined if any individual CpG site from the 450K array was associated with global %-5hmC and evaluated whether those CpGs were also associated with prenatal mercury exposure. After adjusting for covariates and multiple comparisons, we did not observe any significant associations between CpGs correlated with global %-5hmC and prenatal mercury exposure (Figure S5). However, these results must be interpreted with caution, as the genomic inflation for the EWAS of %-5hmC was relatively large ($\lambda = 2.2$), which that could lead to the overestimation of statistical significance (van Iterson et al. 2017). Additionally, a more appropriate study design is needed to address this question, for example, implementing oxidative bisulfite treatment of samples to distinguish individuals CpGs that are hydroxymethylated. Processing and technical batch adjustment for the 450K data have been previously described (Cardenas et al. 2017).

Although these studies measured localized rather than global genomic DNA methylation, they provide evidence that prenatal exposure could potentially affect DNA methylation levels of specific genomic regions. Furthermore, in two animal studies conducted in brain tissue of polar bears (Pilsner et al. 2010) and whole blood from alligators (Nilsen et al. 2016), mercury levels were negatively associated with global DNA methylation of each specific tissue, suggesting that mercury might lead to demethylation of the genome independent of tissue type. In sensitivity analyses, we investigated whether cord blood global %-5hmC or %-5mC were associated with other measures of DNA methylation, such as LINE-1 DNA methylation previously measured in this cohort (Boeke et al. 2012). We did not see any significant or strong correlations between our global epigenetic measures and LINE-1 DNA methylation. Furthermore, prenatal mercury exposure was not associated with LINE-1 cord blood DNA methylation (Figure S6).

The adjusted associations of prenatal mercury exposure with %-5hmC, along with the corresponding increase in the ratio of %-5mC to %-5hmC, were persistent into early childhood but absent in midchildhood, even though our sample size was greater in the latter. This relationship suggests that the 5hmC association with prenatal mercury exposure might be malleable and potentially reversible throughout childhood. This is also supported by our sensitivity analyses, showing that among individuals with repeated epigenomic measurements in cord blood and early childhood ($n = 63$), the association persists, but among individuals with repeated epigenomic measurements in cord blood and midchildhood ($n = 144$), the association does not persist. Global measurements of both 5hmC and 5mC have been shown to decline with age, consistent with our observation of higher levels for both epigenomic measures in cord blood compared to early or midchildhood blood (Buscarlet et al. 2015). However, both epigenomic measurements were not significantly different in early and midchildhood.

Measuring global 5hmC and 5mC content in the genome is innovative in epidemiological studies. Liquid chromatography combined with tandem mass spectrometry has been shown to be among the top two best-performing assays for global DNA epigenomic measurements in a multicenter benchmarking study and observed to most accurately reflect expected differences in global DNA methylation (BLUEPRINT Consortium et al. 2016). Also, our global epigenomic measurements in cord blood might differ from previous studies, as both the proportion of 5hmC and 5mC represent the estimated amount of modified cytosine in the entire genome as opposed to other global assays that only target repetitive DNA elements and are a proxy for global epigenomic modifications. For example, we did not see any significant associations between our global measurement of 5hmC and 5mC with either LINE-1 cord blood DNA methylation or the global distribution of CpG methylation measured with the 450K array. This is not unexpected, as both LINE-1 and the 450K array provide methylation measurements at very distinct genomic positions and have limited coverage of all CpGs present in the human genome. In contrast, our liquid chromatography method has a true global genomic coverage.

However, an important limitation of this method is the lack of information on localized methylation or hydroxymethylation. Given the previously characterized dual role of 5hmC in stem cells, it would be beneficial to measure 5hmC with greater resolution in order to characterize its role in transcriptional regulation and response to environmental exposures at specific genomic coordinates within DNA samples. Another important limitation of this study is that both epigenomic measurements were taken from cord blood and blood DNA, and not isolated from a target organ, such as the brain. Given the cell type specificity of 5hmC and 5mC, our measurement represents an aggregate of different nucleated blood cell types. However, we did adjust for major cell type distributions, including nRBCs found in cord blood, estimated from DNA methylation arrays shown to have good correlations with laboratory measurements (Cardenas et al. 2016). Our cell type adjustment minimizes the chance that our findings are due to cell type heterogeneity. Our study has several strengths, including the relatively large number of samples, the prospective assessment of both epigenomic measurements at birth and at two time points during childhood, and the use of an objective biomarker of prenatal mercury exposure at a sensitive window of fetal development during pregnancy. We used second trimester maternal RBC-Hg concentrations as an unbiased biomarker of exposure with an estimated half-life of 72 d (Mahaffey et al. 2004), and 70-95% of the red blood cell mercury content is estimated to be methylmercury (Mortensen et al. 2014). In our cohort, we also expect that the major source of exposure is dietary, mainly from fish, although we cannot rule out other sources. Although this is an observational study and confounding cannot be ruled out, we adjusted for many different confounders, including important prenatal maternal nutritional information, that have been characterized and validated in our cohort.

Conclusion

Our study is the first to show that prenatal mercury exposure, a global persistent environmental contaminant, is associated with lower 5hmC levels in cord blood DNA, and this association persists in early childhood blood, but likely not beyond that time period. Levels of 5hmC play a critical role in embryogenesis and cell lineage commitment, and are now appreciated as an independent epigenetic control mechanism of transcriptional regulation. The prenatal toxicity of mercury might be mediated by the observed alteration in 5hmC content serving as biomarker of exposure or disease susceptibility. Furthermore, our results highlight the potential role of prenatal mercury exposure during fetal epigenetic programming and suggest that this association is reversible in childhood.

Acknowledgments

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